Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4243–4247, July 1982
Biochemistry

Purification of the cdc8 protein of *Saccharomyces cerevisiae* by complementation in an aphidicolin-sensitive *in vitro* DNA replication system

[permeabilized cells/yeast/cell division cycle (*cdc*) mutants/DNA elongation/eukaryotic DNA replication]

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Communicated by Norman Davidson, July 27, 1981

ABSTRACT **DNA synthesis *in vitro* in Brij-treated *Saccharomyces cerevisiae* requires the product of the *CDC8* gene (Hereford, L. M. & Hartwell, L. H. (1971) *Nature (London) New Biol.* 234, 171–172). Extracts of wild-type A364a yeast restore DNA synthesis in Brij-treated *cdc8*, a mutant containing a thermolabile *cdc8* gene product. This constitutes a complementation assay by which the *cdc8* gene product can be monitored during purification. A heat-stable protein responsible for this complementation has been partially purified from both wild-type A364a cells and from a *cdc8* temperature-sensitive mutant. The complementation activity from the mutant is thermolabile when compared to the wild-type activity, indicating that *CDC8* is the structural gene for the protein.**

The microbial eukaryote *Saccharomyces cerevisiae* is an ideal organism for probing the enzymatic mechanisms of DNA replication in higher cells. While displaying most of the features of higher eukaryotic chromosome structure observed during gene expression and DNA replication, yeast offers the advantage over higher cells of rapid growth and relatively simple organization. In addition, yeast cells can exist stably in both haploid and diploid states, thus facilitating the isolation of mutants and their genetic analysis. Tremendous progress has been made in elucidating the mechanism of DNA replication in bacteria, by analysis of replication mutants and by biochemical characterization of replication components. In these studies an important advance was the development of *in vitro* replication systems that allow purification of components of the complex replication machinery by complementation analysis. Such a combined genetic and biochemical approach for dissecting the replication machinery should also prove profitable in yeast.

Many temperature-sensitive mutants having defects in cell division have been isolated and characterized by Hartwell (1). Of these, several were found to be deficient in DNA synthesis, although only in *cdc8* mutants was it clear that the defect directly affected DNA replication (1, 2). We have examined the existing temperature-sensitive strains for mutants specifically defective in DNA replication, by assaying for replication *in vitro* in yeast cells made permeable to nucleoside triphosphates by the nonionic detergent Brij, as described by Hereford and Hartwell (3). Brij-treated cells retain the morphology of intact cells but are permeable to low molecular weight precursors of macromolecular synthesis. DNA synthesis in this system corresponds to *in vivo* replication by several criteria. In particular, DNA synthesis in the *S. cerevisiae* temperature-sensitive replication mutant *cdc8* is heat labile in this *in vitro* system (3). In addition, as shown in this communication, synthesis is inhibited by aphidicolin, an inhibitor of DNA replication *in vivo*. Using this screening procedure, we have identified nine additional replication mutants (unpublished).

It was not possible to predict *a priori* if detergent-treated cells would be permeable to macromolecules and thus amenable to *in vitro* complementation. During the course of these studies, we investigated whether Brij-treated cells of any of the DNA replication mutants could be complemented by extracts containing the wild-type gene product. *cdc8* alone showed complementation; DNA replication in Brij-treated *cdc8* cells could be restored by the addition of wild-type extract at the restrictive temperature. The ability to achieve complementation with the *cdc8* defect may, in fact, be due to the unique physical properties of the cdc8 protein described here.

In this communication we report the partial purification and characterization of the cdc8 protein, using a complementation assay to monitor activity. This is just the first step in ultimately generating a molecular description of eukaryotic replication by use of *in vitro* complementation to purify and to characterize the gene products involved.

MATERIALS AND METHODS

Strains. Parental strain A364a (*a ade1 ura1 gal1 tyr1 his7 lys2*) and strain 198 *cdc8*, derived from A364a, were provided by John Scott (University of California at Los Angeles).

Medium. YPD (yeast extract/peptone/dextrose) medium is described in ref. 4.

Preparation of Receptor. Strain *cdc8* was grown at 23°C to a density of 2×10^7 cells per ml in YPD medium, collected by centrifugation, and washed twice with H_2O. The cells were suspended in 10 mM Tris·HCl (pH 7.2)/2 M sucrose/1% Brij 58 to a final concentration of 2×10^8 cells per ml and incubated at 30°C until the cells were permeable, as monitored by measuring alkaline phosphatase activity (3). The receptor preparations were prepared fresh for each assay and retained activity at 0°C for 2–3 hr.

Complementation Assay for cdc8 Activity. The standard DNA replication reaction mixture contained 50 mM Tris·HCl (pH 8.0); 10 mM $MgCl_2$; 1.5 mM 2-mercaptoethanol; 50 μM dATP, dGTP, and dCTP; 2 μM [α-^{32}P]dTTP (4,000–8,000 cpm/pmol); 1 mM ATP; 10 mM phospho*enol*pyruvate; 1% Brij 58; 0.05 ml of the Tris/sucrose/Brij cell suspension; and the indicated amounts of donor fractions. After incubation for 30 min at 37°C the reaction was stopped by the addition of 3 ml of 1 M HCl/0.02 M sodium pyrophosphate, and the amount of trichloroacetic acid-insoluble radioactive material was determined (5). A unit of cdc8 activity is that amount of protein giving rise to incorporation of 1.0 pmol of total nucleotide in 30 min.

Other Methods. Protein was measured by the method of Lowry *et al.* (6).

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RESULTS

DNA Synthesis in Permeabilized Yeast Cells Mimics DNA Replication *in Vivo*. *DNA synthesis is thermosensitive in the thermolabile mutant* cdc8. Previous studies by Hereford and Hartwell (3) showed that yeast cells made permeable with Brij 58 could incorporate [α-^{32}P]dTTP into acid-insoluble material in a reaction requiring Mg^{2+}, ATP, and the other three dNTPs. The *in vitro* replication system apparently used the same synthesizing machinery as DNA replication *in vivo*, because DNA synthesis was defective in Brij-treated strain *cdc8* at the restrictive temperature. As shown in Fig. 1, we have reproduced this finding, using a modified procedure that involves more extensive treatment with detergent. The optimal time of Brij treatment is 40 min, a time at which maximal amounts of alkaline phosphatase are also measured. Whereas wild-type cells respond to the detergent treatment by supporting DNA synthesis when provided with dNTPs, no DNA synthesis over background is observed with Brij-treated *cdc8* cells. The level of synthesis in wild-type strain A364a is 5-fold higher than that observed in the mutant *cdc8* at 37°C. Residual synthesis in *cdc8* probably does not represent replication because ATP is not required.

Synthesis is inhibited by aphidicolin. Aphidicolin, a tetracyclic diterpenoid, has recently been shown to inhibit the growth of eukaryotic cells and virus-infected cells by inhibiting DNA replication (7, 8). Furthermore, Plevani (9) has shown that both yeast DNA polymerase I and DNA polymerase II are inhibited by aphidicolin, and Sugino *et al.* (10) have shown that in yeast mutants that are permeable to aphidicolin the drug inhibits growth.

Because inhibition of DNA synthesis by aphidicolin provides one additional criterion for DNA replication, we have examined its effect on DNA synthesis in Brij-treated yeast cells. As shown in Fig. 2, aphidicolin inhibits DNA synthesis in the permeabilized cell system; at a concentration of 20 μg/ml, DNA synthesis is reduced to 40% of normal. However, the addition of increasing amounts of aphidicolin up to 100 μg/ml does not eliminate this residual synthesis. This drug-resistant synthesis may reflect the fact that dNTPs are competitive inhibitors of aphidicolin (8) or may indicate the existence of a previously undetected aphidicolin-resistant DNA-synthesizing system in yeast.

Extracts of Wild-Type Yeast Restore DNA Synthesis in Permeabilized Cells of *cdc8*. Cell-free extracts prepared from a wild-type A364a (donor extract) can restore synthesis to *cdc8* cells made permeable by Brij treatment (receptor). As shown in Fig 3*a*, extracts of wild-type A364a stimulate DNA synthesis in permeabilized cells of *cdc8* at 37°C. In contrast, donor extracts from strain *cdc8* cannot restore the activity to the recipient *cdc8* preparation at 37°C (Fig. 3*a*). The difference in stimulatory activity in extracts from wild-type and *cdc8* indicates that the donor extract is specifically providing the *cdc8* gene product.

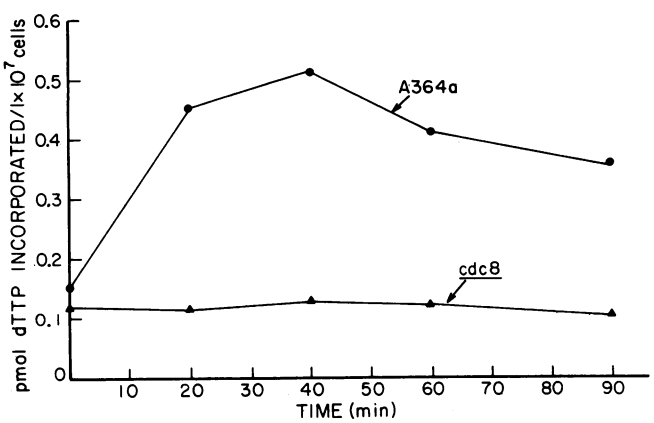

FIG. 1. Incorporation of [^{32}P]dTTP into DNA at 37°C plotted as a function of time of pretreatment for cultures of wild-type A364a and mutant *cdc8*. Strains were grown, harvested, and treated for the indicated time period with Tris/sucrose/Brij. DNA synthesis was then measured in the standard assay using [α-^{32}P]dTTP.

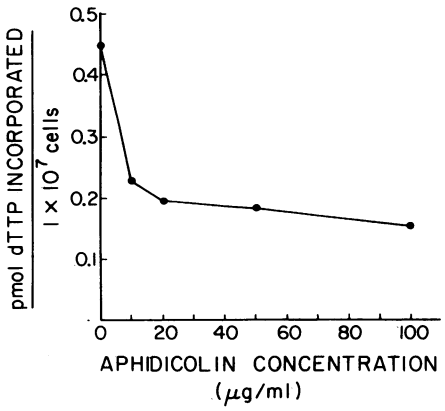

FIG. 2. Effect of aphidicolin on DNA synthesis in permeabilized A364a cells. DNA synthesis in Brij-treated A364a cells was measured with the addition of indicated amounts of aphidicolin dissolved in dimethyl sulfoxide.

The inability of an extract of *cdc8* cells to restore DNA synthesis to permeabilized *cdc8* cells at 37°C is not due to nonspecific loss of activity. When assayed at the permissive temperature, *cdc8* extracts can stimulate DNA synthesis in permeabilized *cdc8* cells (Fig. 3*b*). Thus temperature-sensitive mutant extracts contain a thermolabile gene product. The *cdc8* gene product in the permeabilized cells is apparently more labile than in the extract, perhaps because the Brij-treatment is carried out at 30°C, whereas the extracts are prepared at 4°C. This is an important observation, because the ability of *cdc8* extracts to serve as donors in the complementation assay at 23°C has allowed us to purify a thermolabile activity from the temperature-sensitive mutant.

These observations formed the basis for the complementation assay described in *Materials and Methods*. The requirements for the complementation reaction using partially purified cdc8 protein as donor are summarized in Table 1. In addition to a requirement for both receptor cells and a donor preparation, there is an absolute requirement for ATP. Complementation is inhibited by aphidicolin, just as synthesis is inhibited in wild-type permeabilized cells. A receptor extract prepared from a DNA replication mutant that falls in a different complementation group from *cdc8*, *ts12* (unpublished results), was not complemented in these permeabilized cells. This result not only shows the specificity for *cdc8* in this reaction, but suggests that this may not be a generally useful assay to isolate replication proteins. In permeabilized diploid cells, a 2-fold increase in overall DNA synthesis was routinely observed.

Partial Purification of the cdc8 Protein. Because the *cdc8* gene product is required for DNA synthesis in permeabilized yeast cells, we have been able to purify the cdc8 protein by using the complementation assay described above. The results of a typical purification are summarized in Table 2.

Analysis of glycerol gradient fractions by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that fraction V is greater than 25% pure by weight and

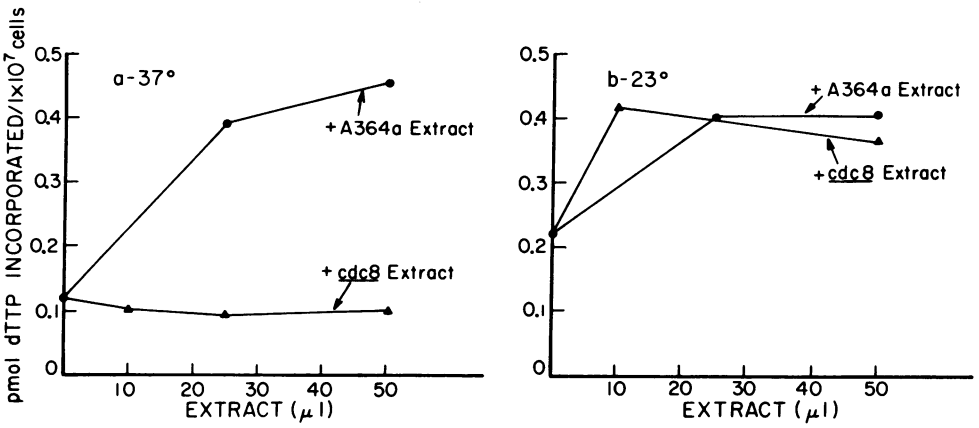

FIG. 3. Complementation of Brij-treated *S. cerevisiae cdc8* cells. Mutant *cdc8* was grown at 23°C to a density of 2×10^7 cells per ml and permeable cells were prepared (receptor). The standard complementation reaction mixture contained receptor cells and the indicated amounts of either wild-type (●) or *cdc8* (▲) extract. Incubation was for 30 min at either 37°C (*a*) or 23°C (*b*).

substantially purer on a molar basis. The glycerol gradient data allow a preliminary estimate of the sedimentation constant of the cdc8 protein that is consistent with a molecular weight (assuming it is a globular protein) of 10,000–20,000, which is also in reasonable agreement with the gel electrophoresis results.

Properties of the Purified cdc8 Protein. *Purified cdc8 protein can complement permeabilized* cdc8 *cells.* Although the cdc8 protein could be purified from extracts by virtue of its ability to restore DNA synthesis to permeabilized *cdc8* cells, the purified protein is much more efficient than crude preparations in stimulating synthesis. As shown in Fig. 4, 25 μg of *cdc8* protein leads to a 10-fold stimulation of activity in the complementation assay at 37°C. The stimulatory activity is linear for amounts of cdc8 protein ranging between 2.0 and 10 μg in the assay. Saturating amounts of cdc8 protein give a 30-fold stimulation of activity (data not shown). Results presented in Fig. 3, however, showed that only a 5-fold stimulation could be achieved with saturating amounts of extract. This is easily explicable if an inhibitor is removed during purification.

Complementation activity of the cdc8 protein purified from the temperature-sensitive mutant cdc8 *is thermolabile.* The cdc8 protein was also purified from extracts of *S. cerevisiae cdc8* through the phosphocellulose step as described above. The purification was monitored by complementation assay at 23°C, a temperature at which the cdc8 protein from this strain complements the mutant extract effectively (see Fig. 3*b*). When fraction IV was assayed at 37°C, however, complementing activity was abolished (Table 3). This is in sharp contrast to the wild-type protein, which has the same activity at 23°C and 37°C. Because the preparation from strain *cdc8* is thermolabile, the complementation activity is the product of the structural gene for *cdc8*.

Complementation by partially purified cdc8 protein is aphidicolin sensitive. After addition of aphidicolin, synthesis in the complementation assay using wild-type cdc8 protein was reduced to 40%, a value similar to that obtained with permeabilized A364a (compare Fig. 2). This argues for, though does not alone establish that, replicative synthesis occurs in this assay.

Heat stability of the cdc8 activity. As shown in Table 4, the activity restoring DNA replication in permeabilized *cdc8* at

Table 1. Requirements of the complementation assay

Omissions and additions	DNA synthesis, pmol dTTP incorporated/per 10^7 cells
Complete	0.61
Omit dNTP	<0.1
Omit ATP	0.12
Omit Mg^{2+}	<0.1
Add aphidicolin (20 μg/ml)	0.23
Omit *cdc8* receptor	<0.1
Omit *cdc8* gene product	<0.1
Omit *cdc8* receptor, add *ts12* receptor	<0.1

The assay conditions were as described in *Materials and Methods*, with the omissions and additions noted. Each reaction mixture (except the second-to-last) contained 10 μg of partially purified cdc8 protein.

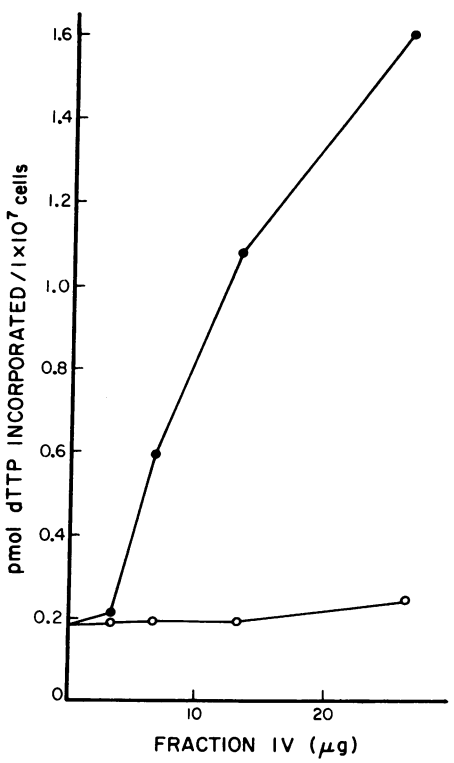

FIG. 4. Complementation by purified cdc8 protein and inhibition by proteinase K. Receptor *cdc8* cells were prepared, indicated amounts of cdc8 protein (fraction IV) were added, and incubation was carried out as in the legend to Fig. 3. The lower curve (○) represents reaction mixtures that contained cdc8 protein that had been treated with proteinase K (100 μg/ml) for 30 min at 65°C.

Table 2. Purification procedure and results of a typical purification

Fraction	Step	Total protein, mg	Specific activity, units/mg	Recovery, %
I	Crude extract	166	4.7	100
II	Streptomyocin and $(NH_4)_2SO_4$	73.7	14.2	134
III	DEAE-cellulose	22.7	52.0	150
IV	Phosphocellulose	1.36	924.4	160
V*	Glycerol gradient	0.04	2900	13.5

Growth, storage of cells and preparation of donor extracts. Strains A364a and *cdc8* were grown at 23°C to late logarithmic phase in YPD medium, collected by centrifugation, washed twice with H_2O, and stored in liquid nitrogen. All subsequent procedures were carried out at 0–4°C. For preparation of extracts, cell paste was thawed, diluted with a solution of Zymolyase 60,000 (0.5 mg/ml in H_2O), and adjusted to OD_{590} = 400. The cells were incubated at 30°C for 30 min, at which time spheroplast formation was complete. The spheroplasts were mixed on a Vortex mixer with glass beads and then disrupted in a chilled homogenizer. The supernatant was then collected by centrifugation at 10,000 × *g* for 40 min (fraction I). *Streptomyocin and ammonium sulfate fractionation.* A solution of 30% streptomyocin sulfate was added to extracts to a final concentration of 5%. After stirring for 30 min, the suspension was centrifuged at 20,000 × *g* for 20 min. Solid $(NH_4)_2SO_4$ (0.47 g/ml) was added to the supernatant fluid and the protein precipitate was collected by centrifugation. The precipitate was dissolved in buffer A [20 mM Tris·HCl (pH 7.6)/1 mM EDTA/1 mM 2-mercaptoethanol/20% (wt/vol) glycerol] and dialyzed against the same buffer (fraction II). *DEAE-cellulose column chromatography.* Fraction II (0.8 ml) was diluted with 0.8 ml of buffer A and applied to a 5-ml DE52 (Whatman) DEAE-cellulose column equilibrated with buffer A. The column was washed and then eluted with a 50-ml linear gradient of NaCl (0.1–0.6 M) in buffer A. Fractions containing the complementation activity were pooled and precipitated by the addition of ammonium sulfate (0.47 g/ml). The precipitate was collected by centrifugation, resuspended in 0.5 ml of buffer B [20 mM Tris·HCl (pH 7.0)/1 mM EDTA/1 mM 2-mercaptoethanol/20% (wt/vol) glycerol] and then dialyzed (fraction III). *Phosphocellulose P-11 column chromatography.* Fraction III was diluted with 0.5 ml of buffer B and applied to a 4-ml phosphocellulose P-11 (Whatman) column equilibrated with buffer B. The column was washed and protein was eluted with a 50-ml linear gradient of NaCl (0.1–0.6 M) in buffer B. Fractions containing complementation activity, which eluted at 0.13 M NaCl, were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, redissolved in buffer C (the same as buffer A except that 0.1 mM dithiothreitol is substituted for 2-mercaptoethanol), and dialyzed against the same buffer (fraction IV). *Glycerol gradient sedimentation.* Fraction IV was dialyzed against buffer C containing 5% (wt/vol) glycerol and layered on a 4.6-ml linear gradient of 10–30% (wt/vol) glycerol containing 0.02 M Tris·HCl (pH 7.5), 10 mM 2-mercaptoethanol, and 10 mM NaCl. Centrifugation was at 45,000 rpm at 2°C for 28 hr in a Spinco SW 50.1 rotor. The following proteins were used as standards: alcohol dehydrogenase, bovine serum albumin, hemoglobin, and cytochrome *c*.

* This step was performed on only a portion of fraction IV and the values in the table were obtained by multiplying by the appropriate factor.

37°C was highly stable, being resistant to incubation at 65°C. After 10 min at 65°C, 80% of the activity remained.

cdc8 activity resides in a protein. The lack of activity in fractions from *cdc8* extracts at the restrictive temperature, shown in Table 3, indicates that the activity resides in a protein or RNA. The activity is resistant to RNase A, however, ruling out that it is an RNA molecule. The activity is also resistant to papain, even at 65°C, a property possibly related to its resistance to heat denaturation (Table 4). However proteinase K, a protease generally considered to be more active toward proteins in native configurations than other proteases, does inactivate cdc8 activity (Table 4, Fig. 4). Controls showed that proteinase K had no effect on synthesis in permeabilized wild-type A364a cells under the conditions in which it inhibited *cdc8* complementation.

Table 3. Thermolability of cdc8 protein from *cdc8* cells

Protein source	DNA synthesis, pmol dTTP incorporated per 10^7 cells: Assay at 23°C	Assay at 37°C	Activity ratio, 37°C/23°C
Wild-type	1.2	1.2	1.0
cdc8	0.80	0.11	0.13

Fraction IV (10 μg) was assayed at 23°C and 37°C in the complementation assay. There was no measurable activity at 37°C when protein from strain *cdc8* was added.

DISCUSSION

The cdc8 protein is essential for DNA replication *in vivo* and is required for cell division. When strain *cdc8* grown at the permissive temperature, 23°C, is shifted to the restrictive temperature, 36°C, cells accumulate with a nucleus that remains undivided but is located at the isthmus between parent cell and bud. The first wave of DNA synthesis after shift up does not occur (1). The product of the *cdc8* gene is apparently required throughout the period of DNA synthesis, because when a synchronized culture of cells defective in the gene is shifted to 36°C within the S period, nuclear DNA replication ceases immediately (2). Mitochondrial DNA replication also ceases at the nonpermissive temperature in *cdc8* mutants (11). Electron microscopic examination of chromosomes in *cdc8* cells placed at the nonpermissive temperature for approximately two generation times shows a high proportion of molecules that contain replication "bubbles." The "bubbles" contain two double-stranded branches with an average length of 3 μm (12).

The formation of replication intermediates at the nonpermissive temperature suggests that the *cdc8* mutant can initiate DNA synthesis at the nonpermissive temperature, while the small size of these "bubbles" may indicate a reduced rate of chain propagation. Thus, the *in vivo* results suggest a role of the cdc8 protein at the replication fork.

Another interesting property of *cdc8* mutants is that DNA extracted at the nonpermissive temperature also contains clusters of small denatured regions, approximately 300 base pairs in length (13). Denatured regions appear on both newly replicated and unreplicated DNA, and disappear upon centrifugation in CsCl, suggesting that the DNA in this region may be

Table 4. Stability of purified cdc8 protein

Treatment	Activity remaining, %
65°C, 10 min	80
Papain at 20 μg/ml, 37°C, 30 min	94
Papain at 20 μg/ml, 65°C, 30 min	76
Proteinase K at 100 μg/ml, 37°C, 30 min	51
Proteinase K at 100 μg/ml, 65°C, 30 min	<1
RNase A at 50 μg/ml, 37°C, 30 min	97

Purified cdc8 (fraction IV) was subjected to various treatments. Treatment with RNase or papain was performed in 0.1 M Tris·HCl (pH 7.0). The partially purified protein activity was then determined by assay as described in the legend to Fig. 1, using 10 μg of fraction IV and with the indicated additions. All values cited are relative to controls carried through identical procedures except for the omission of the indicated agents. The control for heat treatment was maintained at 0°C.

noncovalently bound to protein (16). Such single-stranded regions could be envisioned as intermediates in recombination or replication. Biochemical studies to date indicate that *cdc8* strains are not deficient in precursor synthesis (3). Furthermore, *cdc8* does not code for one of the yeast DNA polymerases (14, 15).

Our findings agree with those of Hereford and Hartwell (3) that incorporation of dNTPs into permeabilized yeast cells corresponds to DNA replication *in vivo*. One of the strongest arguments for this is the finding that Brij-treated *S. cerevisiae cdc8* is incapable of carrying out DNA synthesis at the nonpermissive temperature. Also, we have shown that the synthesis is sensitive to aphidicolin, an inhibitor of yeast replication (9, 10). One point that is not resolved is whether nuclear or mitochondrial replication is being observed, because DNA synthesis occurs on the endogenous DNA template. Banks (16) suggests that all synthesis is mitochondrial, whereas our results indicate that some ρ^0 strains, that is, strains without mitochondrial DNA, are just as active *in vitro* as ρ^+ strains (unpublished). For the current work, resolution of this question is not important, because *CDC8* function is required for both nuclear and mitochondrial replication.

We have extended the earlier studies of Hereford and Hartwell (3) by showing that extracts of wild-type yeast can stimulate DNA synthesis in the permeabilized mutant *cdc8*. This was surprising because the Brij-treated cells retain the morphology of intact cells. The Brij-treated yeast cells are analogous to toluene-treated *Escherichia coli*, which also carry out DNA replication on endogenous templates in a reaction dependent on Mg^{2+} and ATP (17). When toluene-treated *E. coli* are treated with the detergent Triton X-100, DNA polymerase I and lactate dehydrogenase are free to diffuse from the cells, and repair synthesis in the cells is inhibited by antibody to DNA polymerase I (18), indicating that the cells are permeable to macromolecules. We therefore tested whether this yeast system was permeable to proteins by looking for complementation in our mutants and found that *cdc8* showed complementation. We were thus able to use permeabilized yeast cells as receptor extracts in an *in vitro* complementation assay to purify the cdc8 protein.

Using conventional methods of purification, we have purified the cdc8 protein 600-fold. We have shown that *CDC8* is the structural gene for the protein by purifying a thermolabile activity from the temperature-sensitive *cdc8* mutant. Perhaps the most striking property of this protein is its resistance to heating at 65°C and to high levels of papain even at 65°C. Even for proteinase K, a partially denatured protein is required for proteolytic inactivation. Such stability properties are usually attributed to proteins of small size.

What is the function of the cdc8 protein? Results presented here indicate that it participates in DNA replication. In addition, we have recently developed a fully soluble system that carries out replication *in vitro* of exogenous covalently closed circular yeast DNAs that, by many criteria, mimics *in vivo* replication (unpublished results). Synthesis *in vitro* is temperature sensitive in extracts prepared from *cdc8* mutants, and the defect can be complemented by the partially purified cdc8 protein.

Several small heat-stable proteins have been described that participate in replication in prokaryotes. One of them is the *E. coli* helix-destabilizing protein (M_r 18,500) (19, 20). Single-strand DNA-binding proteins are known to participate in events at the replication fork, and a mutation affecting such a protein could give rise to the *cdc8* phenotype. In fact, mutants of *E. coli* that contain a temperature-sensitive helix-destabilizing protein do not grow at high temperature (20). Other small heat-stable proteins have been found in various replication systems [e.g., the M_r 11,000 *E. coli* thioredoxin, a subunit of phage T7 DNA polymerase (21)]. There is also a class of small heat-stable proteins involved in eukaryotic DNA metabolism, namely the histones. Clearly the role of the cdc8 protein will be resolved only when homogeneous preparations of the protein are available.

We thank S. E. Celniker for helpful discussion. This investigation was supported by U.S. Public Health Service Grant GM 25508 and Research Career Development Award CA 00544.

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